PCT/GB 92/01892

28-10-92

The Patent Office

18 October 1992

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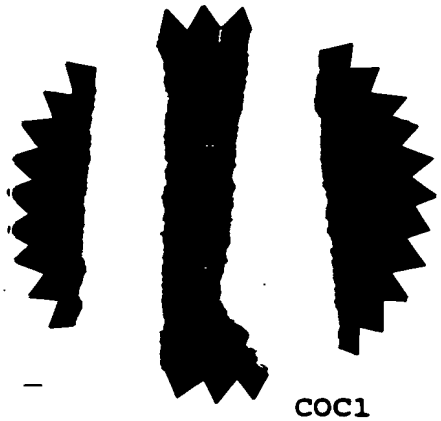

COC1

Signed W. Russell

Dated 15 OCT 1992

K15

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170CT91H00G03450 PAT 1 77 UC 15.00

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The Patent Office

Request for grant of a Patent

Form 1/77

Patents Act 1977

❶ Title of invention

1 Please give the title of the invention: Improved non-competitive sandwich immunoassay system

❷ Applicant's details

❑ **First or only applicant**

2a If you are applying as a corporate body please give:

Corporate name: Multilyte Limited

Country *(and State of incorporation, if appropriate)*: United Kingdom

2b If you are applying as an individual or one of a partnership please give in full:

Surname

Forenames

2c **In all cases**, please give the following details:

Address: Kingsbourne House,
229-231 High Holborn,
London,

UK postcode *(if applicable)*: WC1V 7DA

Country: United Kingdom

ADP number *(if known)*: 5995582001 GB

2d, 2e and 2f: *If there are further applicants please provide details on a separate sheet of paper.*

❑ **Second applicant** ***(if any)***

2d If you are applying as a corporate body please give:

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3a Have you appointed an agent to deal with your application?

Yes [X] No [ ] ➡ ***go to 3b***

⬇ ***please give details below***

Agent's name JY & GW Johnson,

Agent's address Furnival House,
14-18 High Holborn,
London,
WC1V 6DE

Postcode

Agent's ADP number 976001 ✓

3b: *If you have appointed an agent, all correspondence concerning your application will be sent to the agent's United Kingdom address.*

3b If you have not appointed an agent please give a name and address in the United Kingdom to which all correspondence will be sent:

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❹ Reference number

4 Agent's or applicant's reference number *(if applicable)* SGH - Case 10

❺ Claiming an earlier application date

5 Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

Please mark correct box

Yes ☐ No ☐ ➡ ***go to 6***

⬇

please give details below

❑ number of earlier application or patent number

❑ filing date

(day month year)

❑ and the Section of the Patents Act 1977 under which you are claiming:

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15(4) (Divisional) ☐ 8(3) ☐ 12(6) ☐ 37(4) ☐

❻ Declaration of priority

❻ *If you are declaring priority from a PCT Application please enter 'PCT' as the country and enter the country code (for example, GB) as part of the application number.*

Please give the date in all number format, for example, 31/05/90 for 31 May 1990.

6 If you are declaring priority from previous application(s), please give:

Country of filing	Priority application number *(if known)*	Filing date *(day, month, year)*

❼ *The answer must be 'No' if:*
- *any applicant is not an inventor*
- *there is an inventor who is not an applicant,* ***or***
- *any applicant is a corporate body.*

❼ Inventorship

7 Are you (the applicant or applicants) the sole inventor or the joint invento

Please mark correct box

Yes ___ No X ➡ ***A Statement of Inventorship on Patents Form 7/77 will need to be filed (see Rule 15).***

❽ *Please supply duplicates of claim(s), abstract, description and drawing(s).*

❽ Checklist

8a Please fill in the number of sheets for each of the following types of document contained in this application.

Continuation sheets for this Patents Form 1/77			
Claim(s)		Description	9
Abstract		Drawing(s)	2

8b Which of the following documents also accompanies the application?

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Priority documents *(please state how many)*	
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Patents Form 9/77 – Preliminary Examination/Search	
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Improved non-competitive sandwich immunoassay system

The present invention relates to non-competitive sandwich immunoassays. In immunoassays of this type a liquid sample containing an antigen to be assayed such as a hormone is contacted with a receptor (usually an antibody) having binding sites on its molecule specific for the antigen whereby a fraction of the binding sites on the receptor representative of the concentration of the antigen in the liquid sample are occupied by the antigen. The fractional occupancy of the binding sites is then determined by a back-titration technique involving the use of a binding material which is capable of binding with the bound antigen or with the binding sites occupied by bound antigen but not with unoccupied binding sites.

Non-competitive assays are to be distinguished from competitive assays in which the back-titration technique involves the use of a binding material which competes with the antigen for the binding sites on the receptor, for example a labelled version of the antigen or another material able to bind with the unoccupied binding sites on the receptor. In each case the extent of binding of the binding material is determined by the labelling of the receptor on the binding material, for example with a fluorescent label, and comparing the strength of the signal emitted by the bound labelled product of antigen-bound receptor and binding material in the case of the unknown sample with the signal strengths achieved with corresponding samples of known antigen concentration which together provide a dose-response curve. One type of non-competitive sandwich immunoassay involves the use of a labelled receptor and an immobilised binding material, which may or may not be labelled, whereas another involves the use of a labelled binding agent and an immobilised receptor which may or may not be labelled.

It is now well-recognised that non-competitive

labelled receptor sandwich immunoassays generally display higher sensitivity than the more conventional competitive immunoassays which rely upon labelled binding material, usually labelled antigen. The widely accepted explanation for this higher sensitivity is the use of relatively large amounts both of the immobilised receptor (usually an antibody located on a solid support, sometimes known as the "capture" antibody or solid phase antibody) and of the labelled binding material (also often an antibody, sometimes known as the "developing" antibody). By using large amounts of the antibodies, especially the capture antibody, the rate of reaction between antigen and capture antibody is increased, implying in accordance with the law of mass action, that a greater amount of antigen is captured on the solid phase capture antibody in any specified time interval. Thus, the use of large amounts of capture antibody is generally perceived as crucial to the development of non-competitive immunoassays combining very high sensitivity with relatively short incubation times. This approach nevertheless carries disadvantages. For example, it implies heavy consumption of antibodies which may be scarce and costly to produce. It also involves the use of various stratagems to maximise the total surface area of the solid support on which the capture antibody is deposited. For example porous glass microspheres have been used as a solid support in sandwich assay systems, the pores greatly increasing the surface area available for antibody attachment.

Roger Ekins has previously argued, for example in WO-84/01031, WO-88/01058 and WO-89/01157, that this general perception is mistaken and that sensitive and accurate immunoassays are obtainable when and only when the unknown sample and standard samples containing the antigen are each contacted with such a small amount of the receptor that only an insignificant fraction (usually less than 5% and ideally 1-2% or less bearing in mind that errors unavoidably introduced into the measuring procedure elsewhere by limitation in the accuracy of sample and reagent

manipulation, signal measurement, standardisation, temperature variation and the like are generally of the order of 10% or less of the antigen in the sample) becomes bound to the receptor. Only in such circumstances is the fractional occupancy F of the binding sites on the receptor related to the concentration [A] of antigen in the sample (at thermodynamic equilibrium) by the equation

$$F = \frac{K[A]}{1 + K[A]}$$

where K is the affinity constant of the receptor for the antigen and is a constant at a given temperature. Such a technique can be carried out using the receptor spotted onto a solid support in the form of one or more microspots, for example with diameters of 1 mm^2 or less, using sample volumes of the order of ml.

However, with such a system a problem may arise to provide a label which can give a sufficiently strong but sensitive signal. Doubts have also been expressed regarding sensitivities attainable using microspot assay formats on the ground that the use of very small amounts of solid phase antibody must intrinsically necessitate long incubation times and yield low sensitivity.

We have now found that in such a system very good results can be obtained by using as the labelling system micron or preferably sub-micron sized microspheres containing a marker, preferably a fluorescent label. By combining the use of such a label for the developing antibody alone or for both the developing antibody and the capture antibody with very small amounts of capture antibody located at a high surface density on a solid support in the form of a microspot, non-competitive immunoassay systems may be devised which are as rapid to perform as , and possess sensitivities comparable with or indeed greatly superior to, those of conventional sandwich systems relying upon comparatively large amounts of capture antibody. This

crucial finding, which contradicts currently accepted views on the design of high sensitivity immunoassays and is totally unexpected, potentially forms the basis of development of a variety of superior miniaturized immunodiagnostic devices possessing exceedingly high sensitivity whilst requiring only relatively short incubation and measurement times.

According to the present invention there is provided a non-competitive sandwich immunoassay process in which the concentration of an antigen in a liquid sample is determined by comparison with a dose-response curve computed from standard samples, using a receptor having binding sites specific for the antigen and a binding material capable of binding with the bound antigen or with the receptor binding sites occupied by the bound antigen, the receptor being used in an amount such that only an insignificant fraction of the antigen in the sample becomes bound to the receptor and a label being used in the immunoassay in relation to the receptor or the binding material whereby the strength of a signal associated with the label is representative of the fractional occupancy of the receptor binding sites by the antigen, the process being characterised by the use as the label of microspheres having a size of less than 5 μm and containing a marker, preferably a fluorescent label.

Fluorescent microspheres of micron and submicron size have been known since about 1982 and are commercially available under the trade mark FluoSpheres from Molecular Probes Inc.. Suitable microspheres have a diameter of generally less than 5 μm and preferably not more than 1 μm, more preferably of the order of 0.01 to 0.5μm, and it is preferred to use spheres all essentially of the same standard size. The microspheres may be made of any suitable or convenient inert material such as a polymer latex, for example a polystyrene latex, which is desirably provided on its surface with either negatively charged groups such as sulphate, carboxyl or carboxylate-modified groups or positively charged groups such as amidine groups. The

presence of such charged groups on the surface of the spheres allows a wide variety of proteins, such as IgG, avidin/streptavidin and BSA, to be absorbed passively on or coupled covalently to the surfaces of the spheres at various surface densities as desired.

Although the microspheres may contain markers of various types, for example radioactive or chemiluminescent labels, they preferably contain fluorescent labels. Each microsphere desirably contains large numbers of fluorescent dye molecules as labels, for example up to 10 million in 1 μm diameter spheres with smaller numbers in smaller spheres down to about 10 in very small spheres. The fluorescent dye molecules may be selected to provide fluorescence of the appropriate colour range (excitation and emission wavelength) compatible with standard filter sets, for example yellow/green or red. Fluorescent dyes include coumarin, fluorescein, rhodamine, Texas Red, lanthanide chelates and cryptates. Dyes which fluoresce only in non-aqueous media can be used. Preferred fluorescent dyes for use in the microspheres are oil-soluble dyes in order to facilitate their incorporation into the interior of the microspheres. Yellow/green FluoSpheres, which are excited very efficiently at the 488 nm argon laser line, are presently preferred.

In use as the label for the binding material, or for the receptor and the binding material, in the immunoassay systems of the invention the microspheres may have the binding material, or the receptor as the case may be, physically adsorbed onto the surface of the spheres. More conveniently, however, appropriately surface-modified microspheres are selected and the binding material (eg. developing antibody) or receptor (eg. capture antibody) is covalently bonded to them either directly or through a linking molecule such as a carbodiimide. Thus, for example, to link the microspheres and antibody the antibody may be adsorbed onto hydrophobic sulphate-modified microspheres or covalently coupled to aldehyde-modified or carboxylate-

modified hydrophilic microspheres, the latter via a water-soluble carbodiimide. When both the receptor and the binding material are labelled with fluorescent microspheres different dyes will of course be used in the two labels.

The microspheres are primarily used in conjunction with an immunoassay system in which the immobilised material, usually the receptor (capture antibody), is deposited on a solid support in the form of a microspot having an area of 1 mm^2 down to 100 μm^2 or less, for example a diameter of 0.1-01 mm, although for very small microspots it may be necessary to use very small microspheres or fewer larger microspheres. The surface density of the receptor on the microspot is desirably in the range 1,000 to 100,000 IgG/μm^2, preferably 10,000 to 50,000 IgG/μm. These microspots are used in conjunction with sample sizes of 1 ml or less, for example down to 50 or 100 μl or even less depending on the size of the microspot the aim being to cover the microspot.

In other respects the immunoassay may be carried out in a known manner, for example as described in my earlier patent applications as mentioned above or in the literature. It is preferred, although not essential, for both the receptor and the binding material to be antibodies. Monoclonal or polyclonal antibodies may be used and the procedure may be used to assay antigens such as hormones, nucleic acid, proteins, vitamins, drugs or other components of biological samples such as body fluids, the receptor and binding material being appropriately chosen so as to bind to the antigen in question. The so-called antigen can be a nucleotide sequence, in which case the receptor and the binding material may both be other nucleotide sequences, which will differ from one another. The antigens may contain only one epitope for the antibody or the epitope may be replicated on the antigen molecule. The polyclonal developing antibody may react with a variety of epitopes on the antigen or antigen/capture antibody complex or a mixture of two or more monoclonal developing antibodies reacting

Figure 2 which is a graph on the same axes as Figure 1.

Example 3

A dual-label assay was carried out. The developing antibody was conjugated to yellow/green polystyrene latex microspheres of 0.12 μm diameter as described in Example 1. The capture antibody was deposited indirectly on Dynatech Microlite microtitre wells at a surface density of about 40,000 IgG/μm^2 via biotin/avidin. The antibody was first conjugated together with avidin to polystyrene latex microspheres of 0.1 μm diameter containing red fluorescent dye, the conjugated spheres then being allowed to bind to biotin microspots previously coated on the microtitre wells. Although the excitation maximum of the red dye centred at 578 nm, the excitation band was broad enough to permit good signal intensities at 605 nm to be obtained even when exciting at 488 nm with an argon laser, thus allowing the simultaneous scanning of the two dyes. The concentration of antigen (TSH) in the test sample was obtained by observing the ratio of the fluorescent signals from the two dyes and correlating it with the signals using the standard samples.

The results obtained are shown in the accompanying Figure 3 which is a graph of signal ratio against TSH concentration. The sensitivity of the assay (based on measurements of the standard deviation of the zero dose estimate) was 0.0003 mU/litre.

As indicated above, these very high sensitivities for non-competitive immunoassays are unexpected in the light of the currently accepted views on immunoassay design. Some increase in sensitivity would be expected in any immunoassay format due to the use of microspheres because of the increased number of molecules of label attached to each antibody molecule, this resulting in an effective increase in specific activity of the labelled antibody molecules. However, this effect alone would not be expected to result in immunoassay designs departing so markedly from

with different epitopes may be used.

The present invention is illustrated by the following Examples and drawings.

Example 1

An assay for thyroid stimulating hormone (TSH) was carried out using two monoclonal antibodies directed at different epitopes on the TSH molecule as capture and developing antibodies (receptor and binding material), and TSH standard samples supplied by the National Institute for Biological Standards and Control. The capture antibody was deposited as microspots approximately 0.5 mm in diameter on Dynatech Microlite microtitre wells by passive adsorption, giving a surface density of about 40,000 IgG/μm^2. The developing antibody was covalently coupled to carboxylate-modified polystyrene latex FluoSpheres 0.08 μm in diameter containing yellow/green fluorescent dye. The TSH samples were applied to the microtitre wells in amounts of about 200 μl.

Following overnight incubation the results obtained were as plotted on the accompanying Figure 1, which is a graph of fluorescence intensity against TSH concentration in mU/litre. The sensitivity of the assay (based on measurements of the standard deviation of the zero dose estimate) was 0.003 mU/litre.

Example 2

Example 1 was repeated except that the total incubation time was reduced to 1 hour (0.5 hour incubation of sample with capture antibody, followed by 0.5 hour incubation with developing antibody) and the size of the microspheres was increased to 0.12 μm diameter. The sensitivity of the assay was thereby increased ten-fold to 0.0004 mU/litre based on measurements of the standard deviation of the zero dose estimate. The results are plotted in the accompanying

conventional concepts in this field and requiring in particular very small amounts of capture antibody.

Two possible explanations for these unexpected findings can perhaps be advanced. The first is that by confining a very small number of capture antibodies at high surface density to a very small area in the form of a microspot the signal/noise ratios obtained in any finite incubation time may be improved as compared with those obtained in conventional designs in which very large amounts of capture antibody are distributed over large surface areas. The second is that when antigen molecules are located between two solid surfaces on which the capture and developing antibody molecules are respectively located (viz the microspheres and the microtitre well) antigenic sites on the antigen molecules may become bound to multiple developing antibodies if the antigen contains the same epitope replicated on its surface or if the developing antibody is polyclonal or more than one monoclonal antibody directed at different epitopes on the antigen is used, thus increasing the effective affinity of the developing antibody. This implies that the surface density of developing antibody molecules on the microspheres is likely to represent an important determinant of the sensitivities achieved.

Of course, the microspheres can also be used for labelling purposes in a competitive immunoassay system using similarly very small amounts of receptor, but corresponding or substantial increases in sensitivity due to their use would not necessarily be achieved or even expected.

Fig. 1

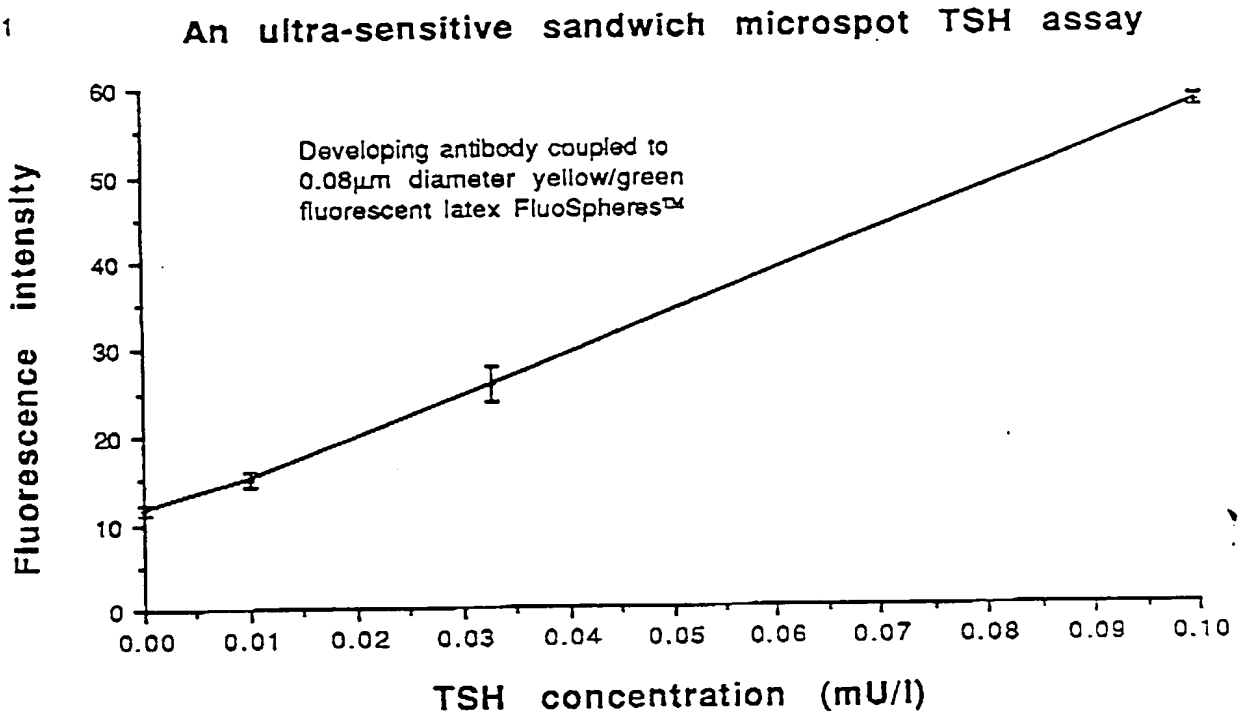
An ultra-sensitive sandwich microspot TSH assay
Developing antibody coupled to
0.08µm diameter yellow/green
fluorescent latex FluoSpheres™
Fluorescence intensity
60
50
40
30
20
10
0
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.10
TSH concentration (mU/l)

Fig. 2

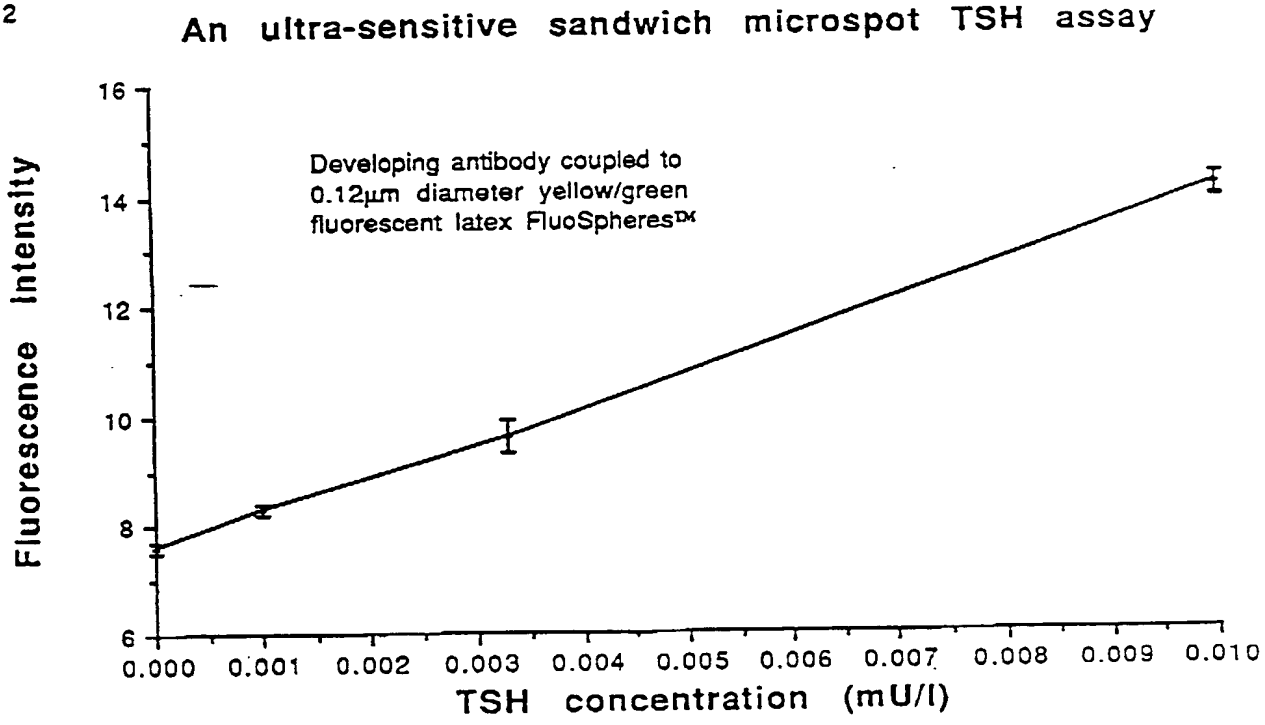
An ultra-sensitive sandwich microspot TSH assay
Developing antibody coupled to
0.12µm diameter yellow/green
fluorescent latex FluoSpheres™
Fluorescence Intensity
16
14
12
10
8
6
0.000
0.001
0.002
0.003
0.004
0.005
0.006
0.007
0.008
0.009
0.010
TSH concentration (mU/l)

Fig. 3

An ultra-sensitive dual-labelled microspot ratiometric TSH assay